Angiogenesis and the atherosclerotic carotid plaque: An association between symptomatology and plaque morphology

Mark J. McCarthy, FRCS(Ed), Ian M. Loftus, FRCS, Matt M. Thompson, MD, FRCS, Louise Jones, MRCPath, Nicholas J.M. London, MD, FRCS, Peter R.F. Bell, MD, FRCS, A. Ross Naylor, MD, FRCS, and **Nicholas P.J. Brindle, PhD,** *Leicester, United Kingdom*

Purpose: **Symptomatic carotid disease resulting from generation of thromboemboli has been associated with plaque instability and intraplaque hemorrhage. These features of the lesion could be influenced by the fragility and position of neovessels within the plaque. The purpose of this study was to determine whether any association exists between neovessel density, position, morphology, and thromboembolic sequelae.**

Methods: **Carotid endarterectomy samples were collected from 15 asymptomatic patients with greater than 80% stenoses and from 13 highly symptomatic patients who had suffered ipsilateral carotid stenotic events within 1 month of surgery. Both groups were matched for gender, age, risk factors, degree of carotid artery stenosis, and plaque size. Samples were stained with hematoxylin/eosin and van Geison. Immunohistochemistry was performed by using an endothelial specific antibody to CD31. Plaques were assessed for histologic characteristics, and neovessels were counted and characterized by size, site, and shape.**

Results: **There were significantly more neovessels in plaques ($P < .00001$) and fibrous caps ($P < .0001$) in symptomatic compared with asymptomatic plaques. Neovessels in symptomatic plaques were larger ($P < .004$) and more irregular. There was a significant increase in plaque necrosis and rupture in symptomatic plaques. Plaque hemorrhage and rupture were associated with more neovessels within the plaque ($P < .017$, $P < .001$) and within the fibrous cap ($P < .046$, $P < .004$). Patients with preoperative and intraoperative embolization had significantly more plaque and fibrous cap neovessels ($P < .025$, $P < .001$).**

Conclusion: **Symptomatic carotid disease is associated with increased neovascularization within the atherosclerotic plaque and fibrous cap. These vessels are larger and more irregular and may contribute to plaque instability and the onset of thromboembolic sequelae. (J Vasc Surg 1999;30:261-8.)**

Internal carotid artery stenosis can give rise to thromboemboli, which can lead to contralateral cerebral ischemic events or ipsilateral amaurosis fugax. These thromboemboli arise from the atherosclerotic plaque and are thought to be more common in soft ulcerated plaques than in calcified or fibrous plaques.[1]

From the Departments of Surgery and Pathology (Ms Jones), RKCSB, University of Leicester.

Supported by grants from the Royal College of Surgeons of Edinburgh research bursary (M.J.M.) and the Royal College of Surgeons of England research fellowship (I.M.L.).

Reprint requests: Mark John McCarthy, FRCS(Ed), Department of Surgery, RKCSB, University of Leicester, Leicester, UK LE2 7LX.

0741-5214/99/$8.00 + 0 **24/1/96863**

Persson et al[2] stated that intraplaque hemorrhage leads to the development of clinical neurological symptoms if this process then ruptures into the luminal surface of the artery. Carr et al[3] described that fibrous cap thinning, foam cell infiltration of the fibrous cap, and an inflammatory infiltrate all predispose the fibrous cap to rupture, for which there were strong correlations with symptomatic carotid artery disease. It appears that plaque instability is a major factor in the development of thromboembolic sequelae.[4] The process of angiogenesis, the development of new blood vessels from previously existing endothelium, has been implicated to play a major role in the development of coronary atherosclerotic plaque hemorrhage,[5] rupture,[6] and thrombosis[5] and thus lead to the progression of coronary artery stenoses[7] to occlusion and myocardial ischemia. What has not been

Table I. Clinical risk factors for symptomatic and asymptomatic patients undergoing carotid endarterectomy

Risk factors	*Symptomatic patients (n = 13)*	*Asymptomatic patients (n = 15)*	*P*
Current smoker	10(77%)	13(87%)	.4
Ischemic heart disease	10(77%)	8(53%)	.2
Hypertension	7(54%)	9(60%)	.7
Hyperlipidemia	4(31%)	4(27%)	.6
Diabetes mellitus	2(15%)	2(13%)	.6

Fisher exact test used to define any statistical difference between the two groups.

established is whether similar events occur in carotid artery plaques and whether patient symptoms correlate with neovessel density within the plaque. It may also be the case that neovessel size, shape, and permeability may also be important determining factors with regard to the development of plaque hemorrhage and rupture.

Therefore, in this study we set out to determine the extent of neovessel formation in carotid plaques that had been removed from patients who had undergone carotid endarterectomy for both asymptomatic and symptomatic carotid artery disease. In addition, we aimed to establish whether there was any association between the presenting symptomatology, used as a surrogate measure of thromboembolic activity, and the number, site, size, and shape of neovessels within the plaque.

PATIENTS AND MATERIALS/METHODS

Endarterectomized carotid plaques were collected from a consecutive cohort of 65 patients who had undergone carotid endarterectomy. From this cohort we identified patients who had been asymptomatic and those who had been preoperatively, highly symptomatic (ie, having suffered a neurological event, 1 month or less before undergoing carotid endarterectomy). Thirteen patients fit into this latter category. Fifteen of the patients who underwent carotid endarterectomy were asymptomatic from neurological events. This allowed comparison of the histologic findings from two sets of patients who were at the extreme ends of the symptomatology spectrum. Preoperatively, these patients had a greater than 70% internal carotid artery stenosis that was identified by means of color-coded duplex ultrasonography. There was no significant difference between the degree of stenosis of symptomatic and asymptomatic patients, by the Mann-Whitney U test (82.6% vs 82%, respectively).

In the symptomatic group, 7 patients were initially seen with less than 1 month's history of transient ischemic attacks (TIAs), one patient had amaurosis fugax, 1 patient a cerebrovascular accident (CVA) with full neurological recovery, and 4 with a history of previous CVA but now with crescendo TIAs. There was no difference between the groups with regard to age; the median age for symptomatic patients was 70 years (58-81 years), and for asymptomatic patients it was 68 years (49-79 years). In the symptomatic patient group there were 10 men and 3 women, and in the asymptomatic group there were 11 men and 4 women. Patient risk factors are summarized in Table I, differences between groups were analyzed by means of the Fisher exact test.

Carotid endarterectomy tissue samples were removed whole with minimal trauma to the plaque and were immediately placed in 4% paraformaldehyde solution. The specimens were then decalcified in formic acid overnight, and transverse sections were routinely processed and embedded in paraffin. From each sample, serial 4 micron sections were stained with hematoxylin and eosin (H&E), elastic van Geison (EVG), and with a monoclonal antibody against the endothelial cell marker CD31 (Sigma, St. Louis, Mo), which is expressed on endothelial cells.

Light microscopy. All tissue samples contained an area of atherosclerotic plaque within the intimal layer, with normal areas of intima adjacent to it. For each plaque, 3 tissue sections were viewed at ×400 magnification by 3 independent observers. All results shown are the mean scores for the quantitative variables. One observer was an experienced histopathologist who assessed the atherosclerotic plaques blindly for rupture, hemorrhage, fibrous cap thinning, necrosis, and the presence of capsular foam cells on H&E- and EVG-stained sections. The artifact of surgical cutting was identified by direct communication of the tract from the periphery to the lumen or within the plaque. Plaque rupture was associated with a thin fibrous cap unrelated to the surgical tract. *Intraplaque hemorrhage* was defined as the presence of blood and thrombus within the plaque when seen microscopically. *Hemorrhage* was used to refer to a hematoma within the carotid plaque that contained all of the blood

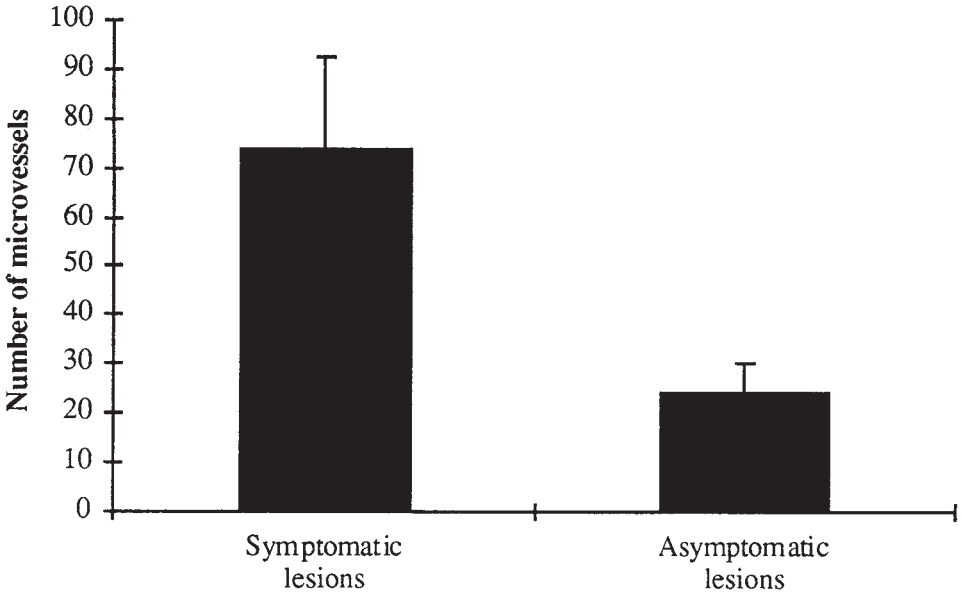

Fig 1. Number of microvessels identified from plaques of symptomatic and asymptomatic patients. Values are medians and interquartile ranges (W = 262.5, $P < .0004$).

elements. *Thrombus* was defined by the presence of platelets or fibrin, characterized by lamination with or without red and white cells interspersed.

The other two observers assessed the samples for the presence of neovessels both independently and blindly. Only blood vessels that appeared to contain a single layer of endothelial cells and no surrounding smooth muscle cells were included. Similarly, only neovessels that contained a lumen were included; however, vessels that contained blood cells but with no endothelial staining were excluded. The number of neovessels throughout the whole of the section were counted, and their position within the plaque was recorded along with their shape and size (calculated as the cross-sectional area of the largest vessel seen in the high-powered field). These data were recorded on a standardized protocol by each observer. The protocol contained a drawing of a representative plaque and surrounding intima onto which the above data could be recorded. The final quantitative data was analyzed as mean scores of the observations of the two observers. There was no statistical difference with regard to interobserver variation.

Statistical analysis. Nonparametric continuous variables are shown as medians (range) and were compared by using the Mann-Whitney U test.[8] Statistical significance was taken as a $P < .05$. Categorical data were analyzed with the Fisher exact test.[8]

RESULTS

The histologic findings of the 13 carotid endarterectomy samples in highly symptomatic patients were directly compared with the 15 samples from asymptomatic patients. There was no difference in carotid plaque section size between the two groups (symptomatic plaques median size, 24 mm^2 [range 8-50 mm^2] vs asymptomatic plaques median size, 23 mm^2 [range 10-45 mm^2], W = 190.5, $P = .9$).

Neovascularization and the carotid plaque. When taking into account plaque size, the median neovessel density (number of vessels per square millimeter) for plaques from symptomatic patients was 4 vessels/mm^2 (range 1.2-9), and for asymptomatic plaques it was 0.7 vessels/mm^2 (range 0-3.8). There were significantly more neovessels present in plaques from symptomatic lesions compared with those that were asymptomatic (W = 262.5, $P < .0004$), as illustrated in Fig 1. The majority of these vessels could be seen at the medial and lateral corners of the plaques, as shown in Fig 2, with fewer being found around the base of the plaque. However, a significant number of symptomatic lesions had neovessels within the atherosclerotic plaque fibrous cap, as illustrated in Fig 3. The median number of vessels within the cap for symptomatic lesions was 12 (range 0-21), versus a median of 0 (range 0-14), for asymptomatic lesions (W = 271, $P < .0001$).

Symptomatic lesions had significantly larger

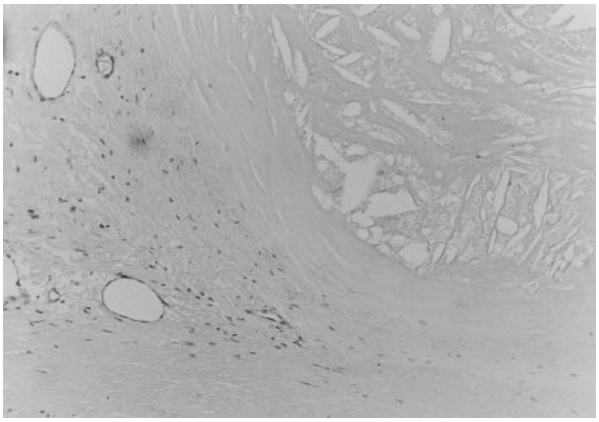

Fig 2. Carotid plaque neovessels at the medial border of the lipid core from a symptomatic patient.

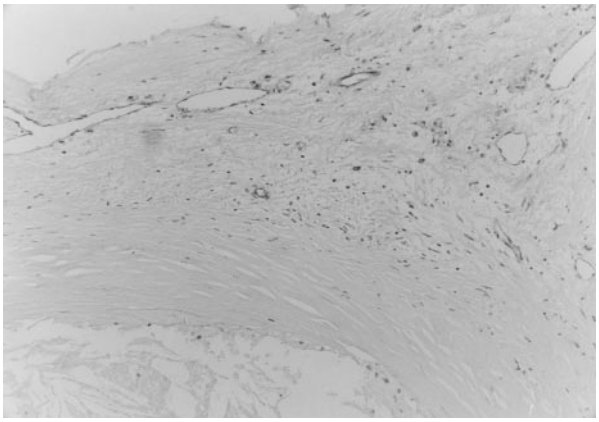

Fig 3. Neovessels within the cap of an atherosclerotic plaque of the carotid artery.

Table II. Carotid plaque histopathologic characteristics from highly symptomatic patients versus those who were asymptomatic at the time of carotid endarterectomy

	Asymptomatic (n = 15)	*Symptomatic (n = 13)*	*P*
Plaque rupture	2(13%)	9(69%)	.010
Intraplaque hemorrhage	3(23%)	8(62%)	.054
Necrosis	7(47%)	11(85%)	.043
Cap thinning	8(53%)	8(62%)	.390
Cap foam cells	11(73%)	11(85%)	.600

neovessels, which were measured by luminal cross-sectional area of the largest vessels identified within the plaque. The median luminal cross-sectional area of the largest neovessels in symptomatic lesions was 0.125 mm^2 (range 0-2.5 mm^2) compared with 0.0025 mm^2 (range 0-150 mm^2) in asymptomatic lesions (W = 248.5, $P < .004$).

It was also noted that there was a large variation in the shape of the neovessels seen. Neovessel shape could be divided into three groups: (1) highly irregular vessels, which appeared multilobular on cross section (Fig 4), (2) circular regular-shaped vessels, and (3) flattened but patent vessels. The number of neovessels falling into each group was determined for each plaque. In the symptomatic lesions the majority of neovessels were irregular in shape 11 lesions (85%), whereas in asymptomatic lesions very few irregular-shaped vessels were identified; 5 lesions had no neovessels present despite looking at further tissue sections; in 7 lesions the majority of vessels were flattened; and in 3 lesions the majority of neovessels were circular and regular in shape.

Neovascularization and plaque morphology. Each plaque was scanned for the presence of rupture, hemorrhage, necrosis, cap thinning, and the presence of capsular foam cells (Table II). For the 28 lesions histologically examined, 11 had the presence of rupture within the plaque, 11 had hemorrhage within the plaque, 17 had cap thinning, 22 had the presence of foam cells, and 18 had necrosis. By the Fisher exact test, there was no significant difference between lesions in symptomatic and asymptomatic patients with regard to plaque hemorrhage, cap thinning, and foam cells. Symptomatic lesions had significantly more necrosis and plaque rupture than did asymptomatic lesions.

Those plaques that ruptured had significantly more neovessels overall than those that did not rupture (W = 225, $P < .0014$), and, similarly, those that ruptured had significantly more neovessels within the cap (W = 219, $P < .0041$). Similar differences were seen in the overall number of neovessels (W = 210, $P < .0168$) and cap neovessels (W = 202, $P < .0469$) that were associated with plaque hemorrhage. Plaque necrosis was also associated with an increase in the number of plaque neovessels (W = 93.5, $P < .0116$) and cap neovessels (W = 88, $P < .0052$). All these results are shown in Figs 5 and 6. Although there were significantly (W = 47.5, $P < .024$) more plaque vessels associated with capsular foam cells (median = 53 [range 0-70]) than when the foam cells were not present (median = 10 [range 0-60]), there was no significant difference with regard to cap vessels and the presence or absence of foam cells (median number of cap vessels in the presence of foam cells = 15 [range 0-70] vs median number of cap vessels in the absence of foam cells = 0 [range 0-18]).

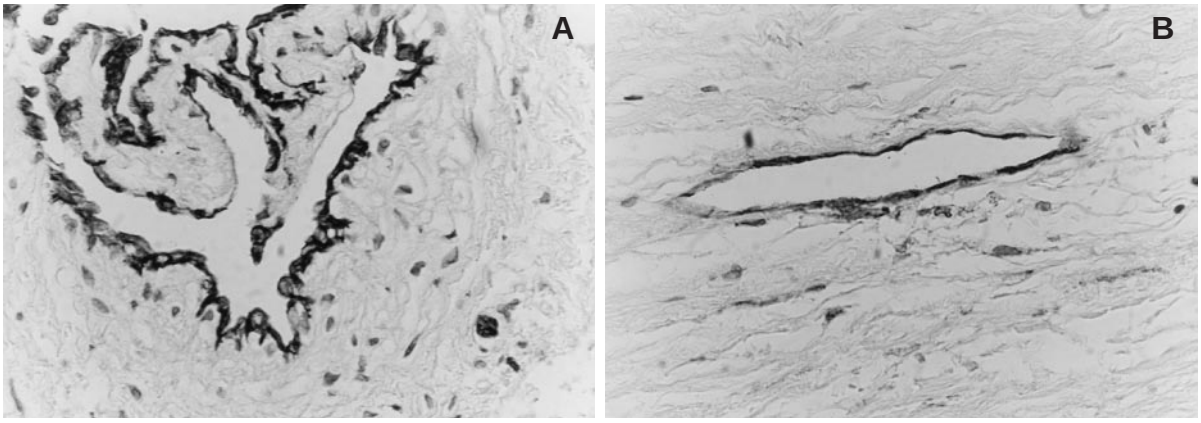

Fig 4. Different neovessel morphology as viewed at 400× magnification. **A,** Irregular lobulated shaped vessel; **B,** flattened vessel.

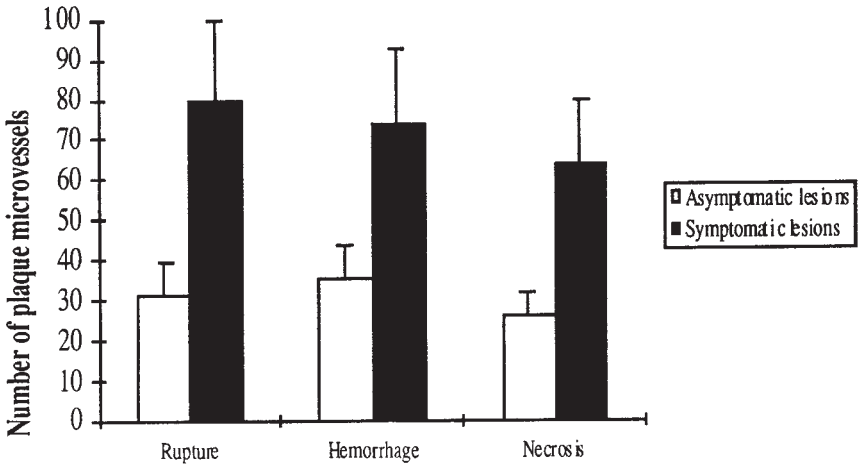

Fig 5. Number of total plaque microvessels associated with plaque, rupture, hemorrhage, and necrosis. Values shown are medians and interquartile ranges.

Transcranial Doppler scanning was performed on all patients at least 30 minutes before carotid endarterectomy, continuously throughout the operation, and then for up to 3 hours postoperatively. For the purpose of this study, only emboli occurring preoperatively and up until the time of carotid cross clamping were included. Of the 28 patients studied, 8 had one or more emboli develop during this period. These emboli occurred in all the symptomatic patients. No asymptomatic patients had any emboli develop during this time course. When the plaque histologic findings of these 8 patients were compared with those of the other 20 in the study, the patients in whom embolization developed had significantly more necrosis within the plaques (88% vs 53%, $P < .016$); however, there was no significant difference between plaque rupture, hemorrhage, cap thinning, and cap foam cells between the groups.

Patients who had embolized had significantly (W = 159, $P < .025$) more plaque vessels (median = 68 [range 32-101]) than did those who did not (median = 31 [range 0-60]). Similar findings were seen for cap neovessels (median number of cap vessels for patients who had embolized = 12.5 [range 7-18] vs median number of cap vessels in those who did not embolize = 0 [range 0-21]: W = 176, $P < .0014$).

DISCUSSION

It has been suggested that intraplaque neovascularization of coronary artery atherosclerotic plaques may be important in the growth of these lesions,[7] and angiogenic blood vessels have been shown to be pre-

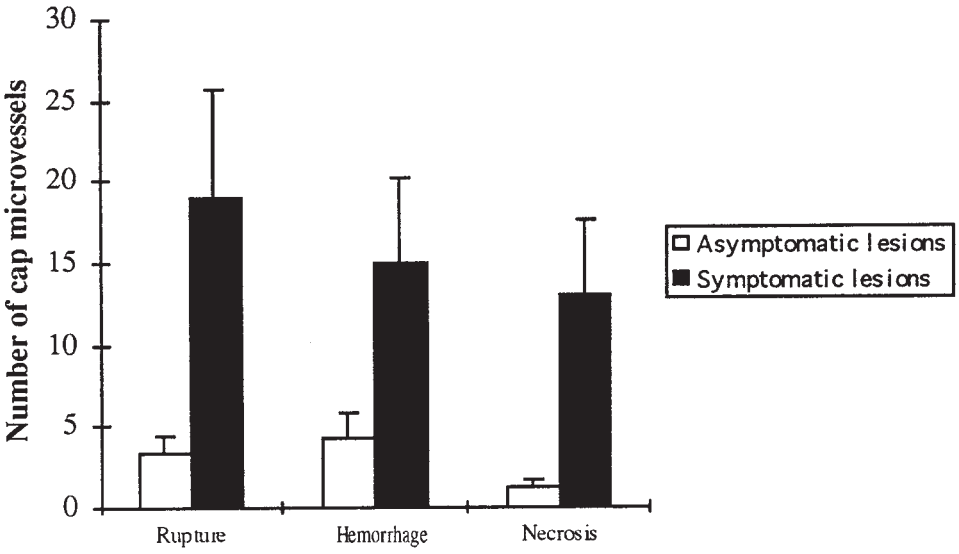

Fig 6. Number of cap microvessels associated with plaque, rupture, hemorrhage, and necrosis. Values shown are medians and interquartile ranges.

sent at the origin of new intraplaque hemorrhage in carotid arteries.[9] The present study has demonstrated that angiogenesis is significantly increased in carotid artery atherosclerotic plaques from symptomatic patients when compared with matched individuals with asymptomatic plaques. In addition, there was an increased number of neovessels seen within the fibrous cap of the plaques of symptomatic patients. Importantly, this difference in neovessel number does not appear to reflect plaque growth as all the plaques gave rise to stenotic lesions that were the same size irrespective of symptomatology. Rather, as symptomatology is likely to reflect thromboembolic activity, these data suggest a correlation between neovessel counts and events at the plaque leading to the generation of neurological symptoms. Furthermore, not only were there more neovessels seen in symptomatic plaques but the overall morphology of these vessels was different than those seen in asymptomatic plaques. The vessels from symptomatic plaques were larger in luminal cross-sectional area and overall were more irregularly shaped. It is possible therefore that these vessels are reminiscent of immature fragile like vessels seen in tumors[10] and mouse embryos deficient of tie-1/tie-2[11] and angiopoietin-1,[12] which are receptors and ligand essential for the latter stages of angiogenesis. If this were so, then large irregular immature vessels present in symptomatic plaques might be more prone to hemorrhage and ultimately rupture. Furthermore, with an increase in neovessel numbers seen in the fibrous caps of symptomatic plaques, this could also be a possible vehicle for delivering inflammatory cells and macrophages to the fibrous cap. It has been demonstrated that increased monocyte/macrophage infiltrate within the fibrous cap is associated with increased plaque instability and patient symptomatology.[13] The cause of this increased instability is thought to be due to breakdown of the extracellular matrix (ECM) caused by matrix metalloproteinase (MMPs) enzymes secreted by the macrophages/foam cells.[4,14] These proteases may ultimately lead to fibrous cap thinning, which has been correlated with increased plaque instability and symptomatology. The origin of fibrous cap foam cells is unknown, but it has been hypothesized that these macrophages are attracted across the luminal surface[13] by chemoattractants released from endothelial and smooth muscle cells within the vessel wall following possible activation by, for example, chlamydia, cytomegalovirus, and other infective agents,[15,16] lipids,[13] and other unknown agents. It is possible that neovessels within the plaque could enhance macroinfiltration by providing an additional entry point and allowing passage of further macrophages and inflammatory cells into the ECM, therefore allowing further release of MMPs and subsequent weakening of the fibrous cap. However, the stimulus for angiogenesis in these plaques is currently unknown, but it may be part of the plaque remodeling process, which has previously been alluded to by Fryer et al. [9] It has been shown that during the development of the aorta, vasa vasorum only appear in the media after this layer has

reached a critical thickness.[17] These findings are similar to the current notion of tumor angiogenesis, in which it is thought that for a tumor to grow above a certain size it needs to develop its own blood supply and therefore tissue ischemia and hypoxia are probably thought to be the stimuli for this process.[18] This could be a similar stimulus for the development of atherosclerotic plaque neovascularization. However, Silverman et al[19] demonstrated that the presence of fat alone could be a stimulus for angiogenesis, and so perhaps the fat-laden lipid core seen in atherosclerotic plaques could act as a stimulus for the growth of new blood vessels. Despite this possibility, it is difficult to explain why five of the lipid-rich plaques from asymptomatic patients had no evidence of angiogenesis occurring within them; so it is difficult to conclude what the stimulus might be. The large irregular immature neovessels may be similar to those seen in tumors, where the hyperpermeability of these vessels leads to tumor edema and hemorrhage. Similar occurrences in carotid plaques could have disastrous consequences on plaque stability and on patient symptomatology.

Carotid plaque rupture,[3] necrosis,[20] hemorrhage,[21] fibrous cap thinning, and the presence of capsular foam cells[3] have all been correlated with onset of neurological symptoms in patients with carotid stenoses. However, from this study only plaque rupture and necrosis were significantly higher in symptomatic patients, while intraplaque hemorrhage was just outside the realms of statistical significance ($P = .054$). Several authors have argued that intraplaque hemorrhage is equally present in both asymptomatic and symptomatic plaques[3,22,23] and that the main difference occurs when the hemorrhage ruptures into the lumen of the carotid artery.[2] Overall, there were significantly increased numbers of angiogenic blood vessels seen in the plaques and fibrous cap that had ruptured, hemorrhaged, and undergone necrosis. Interestingly, there were also significantly more vessels seen in plaques from patients who had evidence of preoperative and intraoperative embolization, embolization being a marker of plaque friability and instability. This demonstrates further the association of plaque instability and angiogenesis. However, whether angiogenesis occurs as part of the remodeling process following plaque rupture and intraplaque hemorrhage of the unstable plaque or that a stable plaque progresses to an unstable plaque as a direct result of neovascularization is yet to be elucidated.

In conclusion, there is an increase in angiogenesis in carotid atherosclerotic plaques from symptomatic patients when compared with plaques from matched asymptomatic patients. Furthermore, the distribution of some of these neovessels within the fibrous cap, the increased size, and irregularity in vessel shape suggest that these vessels could contribute to plaque instability and thus patient symptomatology. However, further prospective studies are required to determine the stimulus for the development of these vessels and their role in plaque progression and remodeling. It is possible that the neurological sequelae of carotid disease could be ameliorated by aiding the maturation and stabilization of plaque neovessels or even preventing plaque angiogenesis.

REFERENCES

1. Sterpetti A, Hunter W, Schultz R. Importance of ulceration of carotid plaque in determining symptoms of cerebral ischemia. J Cardiovasc Surg 1991;32:154-8.
2. Persson A, Robichaux W, Silverman M. The natural history of carotid plaque development. Arch Surg 1983;118:1048-52.
3. Carr S, Farb A, Pearce W, Virmani R, Yao J. Atherosclerotic plaque rupture in symptomatic carotid artery stenosis. J Vasc Surg 1996;23:755-65.
4. Falk E, Shah P, Fuster V. Coronary plaque disruption. Circulation 1995;92:657-71.
5. Kumamoto M, Nakashima Y, Sueishi K. Intimal neovascularisation in human coronary atherosclerosis its origin and pathophysiological significance. Hum Pathol 1995;26:450-6.
6. Patterson J. Capillary rupture with intimal haemorrhage as a causative factor in coronary thrombosis. Arch Pathol 1938; 25:474-87.
7. O'Brien E, Garvin M, Dev R, Stewart D, Hinohara T, Simpson JB, et al. Angiogenesis in human coronary artery atherosclerotic plaques. Am J Pathol 1994;145:883-94.
8. Bland M. An Introduction to Medical Statistics. 2nd ed. New York: Oxford University Press; 1996.
9. Fryer J, Myers P, Appleberg M. Carotid intraplaque hemorrhage: the significance of neovascularity. J Vasc Surg 1987; 6:341-9.
10. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Med 1995;1(1):27-31.
11. Sato TN, Tozawa Y, Deutsch U, Wolburg-Bucholz K, Fujiwara Y, Gendron-Maguire M, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 1995;376:70-4.
12. Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre SD, Davis S, et al. Requisite role of angiopoietin-1, a ligand for the Tie2 receptor, during embryonic angiogenesis. Cell 1996;87:1171-80.
13. Davies M, Richardson P, Woolf N, Katz D, Mann J. Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage and smooth muscle cell content. Br Heart J 1993;69:377-81.
14. Loftus IM, Goodall S, Crowther M, Jones L, Bell PRF, Naylor AR, et al. Increased proteolytic activity in acute carotid plaques—therapeutic avenues to prevent stroke. Br J Surg 1998;85:556.
15. Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? Lancet 1997;350:430-6.
16. Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, et al. A role for macrophages scavenger receptors

in atherosclerosis and susceptibility to infection. Nature 1997;386:292-6.
17. Clark J. An x-ray microscopic study of the postnatal development of the vasa vasorum in the human aorta. J Anat 1965;99:877-89.
18. Folkman J. Tumour angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182-6.
19. Silverman K, Lund D, Zetter B, Lainey JA, Shahood D, Folkman J, et al. Fat angiogenesis: a possible link to coronary atherosclerosis and thrombosis. Circulation 1985;72(Suppl III):282.
20. Feeley T, Leen E, Colgan M, Moore D, O'Brien Hourihane D, Shanik G. Histologic characteristics of carotid artery plaque. J Vasc Surg 1991;13:719-24.
21. Bornstein NM, Krajewski A, Lewis AJ, Norris JW. Clinical significance of carotid plaque hemorrhage. Arch Neurol 1990;47:958-9.
22. Hatsukami T, Ferguson M, Beach K. Carotid plaque morphology and clinical events. Stroke 1997;28:95-100.
23. Van Damme H, Vivario M, Boniver J, Limet R. Histologic characterization of carotid plaques. Cardiovasc Pathol 1994;3:9-17.

Submitted Sep 21, 1998; accepted Jan 4, 1999.